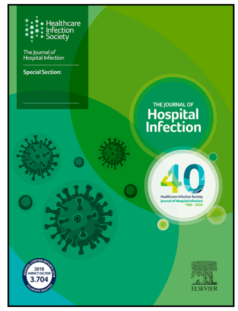

Prevalence of alcohol-tolerant and antibiotic-resistant bacterial pathogens on public hand sanitizer dispensers

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Prevalence of alcohol-tolerant and antibiotic-resistant bacterial pathogens on public hand sanitizer dispensers

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Keywords: Hand sanitizer; antibiotic resistance; alcohol tolerance; bacteria; infection

Abstract:

Introduction:

Since the advent of Covid-19 pandemic, alcohol-based hand sanitizer dispensers (HSDs) are installed in most public and clinical settings for hygiene purposes and convenient application. However, this raised concerns if sanitizer-tolerant bacterial pathogens can colonize on HSDs, which can spread diseases and antibiotic resistance.

Methods:

We conducted sampling from operational automatic HSDs, specifically the dispensing nozzle in direct contact with sanitizer. Culture-dependent cultivation of bacteria and MALDI-TOF were employed to assess microbiological contamination. Bacterial isolates were selected for rapid killing and biofilm eradication assays with alcohol treatment. Antibiotic minimal inhibition concentration (MIC) assays were performed according to the Clinical & Laboratory Standards Institute guidelines. Virulence potential of bacterial isolates was evaluated in the *Caenorhadbitis elegans* infection model.

Results:

Nearly 50% HSDs from 52 locations, including clinical settings, food industry and public spaces, contain microbial contamination at 10^3 - 10^6 bacteria/ml. Bacterial identification revealed *Bacillus cereus* as the most common pathogen (29 %), while Enterobacter cloacae was the only Gram-negative bacterial pathogen (2 %). Selecting *B. cereus* and *E. cloacae* isolates for further evaluation, we found that these isolates and associated biofilms were tolerant to alcohol with survival up till 70%. They possessed resistance to various antibiotic classes, with higher virulence than lab strains in the *C. elegans* infection model.

Conclusion:

HSDs serve as potential breeding grounds for dissemination of pathogens and antibiotic resistance across unknowing users. Proper HSD maintenance will ensure protection of public health and sustainable use of sanitizing alcohols, to prevent emergence of alcohol-resistant pathogens.

Keywords: Hand sanitizer dispenser; bacteria; antibiotic resistance; alcohol tolerance; virulence

Introduction:

Alcohols are commonly used to control microbial infection in clinical settings globally. Due to the Covid-19 pandemic, alcohol-based hand sanitizer dispensers (HSDs) are also installed in various locations, such as home, food and beverage settings, and public spaces. This enabled convenient application by public users for rapid hygiene maintenance. Although manual HSDs remain in use, their hand-operated levers contained most pathogens while dispensing nozzles remain sterile [1]. This drives the increasing use of automatic and contact-free HSDs with sensors that detect the hands placed under the nozzle spout, and dispenser that pumps the alcohol directly onto the outstretched palms, thereby reducing the spread of potential pathogens.

It is noted that hand sanitizers of various brands can kill nearly all pathogens [2], but recent studies had shown that hospital-acquired clinical isolates may gain tolerance to alcohols [3]. Microbial contamination was also found in alcohol manufacturing plants [4]. Mutations in carbohydrate metabolism enable bacteria to survive at higher alcohol concentrations [3]. Formation of multicellular biofilms with their sticky exopolymeric matrix acting as physical barrier can protect bacteria from alcohol killing [5, 6].

This raises an impending question if widespread use of HSDs and similar devices enables the colonization of alcohol-tolerant bacteria, especially in the dispensing nozzle spout in direct contact with alcohol, with a potential to cause the spread of microbial diseases. Our study aims to directly show the presence of bacteria in direct contact with hand sanitizer, with factors accounting for antibiotic resistance, biofilm formation and virulence potential. By swabbing the dispensing nozzle spout from operational automatic HSDs in direct contact with hand sanitizer, we showed the presence of live alcohol-tolerant bacteria with antibiotic resistance and ability to cause diseases in a *Caenorhabditis elegans* infection model, indicating the need to consider microbial contamination in HSDs seriously.

Methods

Microbiological sampling from hand sanitizer dispensers

Ethical approval was granted by the Research Safety Sub-committee, Hong Kong Polytechnic University (ARSA-21134-DEPT-ABCT). Standard microbiological sampling, detection and enumeration of bacteria from swabs were performed in accordance to Public Health England standard methods [7]. Sampling was achieved by swabbing the entire area of mouth opening of the nozzle from working hand sanitizer dispenser by using the sterile 3M™ Quick Swab which contained the Letheen neutralizing buffer used to neutralize disinfectant effect. Samples were collected from 52 local sites in Oct 2021 for examination on the day of collection or within 12 hrs of collection.

Bacterial isolation

Samples were vortexed briefly to aid the release of microbes into the diluent, followed by transfer and spreading on standard petri dishes each containing 20 ml lysogeny broth agar (LBA) for growth of microbes. The petri dishes were incubated in room temperature, where bacterial colony growth was observed every day for 3 days. Colonies with unique phenotype (morphology, shape and colour) are picked for further experiments and stored with 50% (v/v) glycerol at -80 °C.

Matrix-assisted laser desorption-ionisation time-of-flight (MALDI-TOF) mass spectrometry analysis

Per manufacturer's instructions in MALDI Biotyper® Protocol Guide (Bruker Daltonics), a bacterial colony directly smeared as a thin film onto a sample position on a MALDI target plate, then overlaid with 1μl formic acid (70%) and 1μl of HCCA solution + 10 mg/ml of α-cyano-4-hydroxycinnamic acid in standard solution within 30 min and dried at room temperature. Standard solution was prepared with 50 Vol% Acetonitrile, 47.5 Vol% Milli-water (MILLIPAK® 40 GAMMA GOLD) and 2.5 Vol% Trifluoroacetic acid. As previously described [8], the sample plate was performed with MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight mass spectrometry, Bruker Daltonics GmbH & Co. KG), under control by FlexControl ultraflex TOF/TOF software. Each spectrum had a summation of 200 laser shots with a mass range of 2000–20000

Da. The spectrum from each microbe is matched against each main spectrum in the microbe library. The range from 2.000-3.000 indicates the high confidence identification and 1.700-1.999 indicates low confidence identification.

Bacterial colony-forming units (CFU) assay

As previously described [9], bacterial cultures were serially diluted, grown on LB agar and incubated at 37 °C for 16 hrs. Colonies were enumerated and the CFU/ml was tabulated by (no. of colonies x dilution factor) / volume of culture plate.

Alcohol killing assay

Bacterial cells from overnight cultures were washed with 0.9% NaCl (w/v) saline and their OD600nm was adjusted to 0.3 in LB containing various concentrations (0%, 4.38%, 8.75%, 17.50%, 25%, 35% and 70%) of ethanol. For rapid killing with ethanol, the bacterial cells were incubated at 37 °C for 10 mins. The bacterial populations were enumerated with CFU assay as described above.

Biofilm eradication assay

As previously described [10], the minimal biofilm eradication concentration (MBEC) assay (previously known as Calgary Biofilm device) was employed by using Nunc Immuno TSP Lids (Thermo Scientific™). The bacterial isolates were cultivated in 200 µl LB media to enable biofilm formation on the peg surfaces at 37 °C for 24 hrs. After washing the biofilms three times with 0.9% NaCl (w/v) saline, mature biofilms on the peg lids were fitted into 96-well microtitre plates containing 6 different concentrations of ethanol (70%, 35%, 17.5%, 8.75%, 4.375%, and 0%). After 24 hrs at 37 °C, biofilm cells were disrupted into saline by sonication in ice water bath for 10 mins, followed by 15 secs rigorous vortexing for 3 times. For quantification of bacterial numbers, CFU assay was employed as described above.

Endospore staining assay

A 10 µl aliquot of bacterial sample was added to the centre of the glass slide, where the sample was airdried for 5 minutes and heat fixed. A few drops of 1% Malachite green stain were added to the fixed sample and steamed for 5 mins. Distilled water was used to wash away the stain, followed by addition of a few drops of safranin to stain bacterial samples for 30 secs. Distilled water was also used to wash away the remaining stain. Representative brightfield images of the bacterial cells and endospores were captured by a brightfield microscope (Zeiss, Germany) under 100X objective.

Antibiotics susceptibility assay

The minimum inhibitory concentrations (MICs) testing of antibiotics on bacterial isolates were determined according to the Clinical & Laboratory Standards Institute guidelines [11-13]. Bacteria were cultivated in 200 µl Mueller-Hinton (MH) media with various antibiotic concentrations in 96-well plate (Thermo Scientific™, Nunc). The OD_{600nm} values of each well were quantified at 0 hr, 8 hrs and 24 hrs with a microplate reader (Tecan Infinite 2000), where the MIC was determined at the antibiotic concentration with no bacterial growth.

C. elegans infection assay

As previously described [14, 15], the Bristol N2 wild-type *C. elegans* provided by the Caenorhabditis Genetics Center, the University of Minnesota was maintained. For nematode killing assay, the bacterial isolates were first cultivated as bacterial lawns on peptone-glucose-sorbitol agar (PGS; 1% Bacto-Peptone/1% NaCl/1% glucose/0.15 M sorbitol/1.7% Bacto-Agar) at 37 °C for 24 hrs [16]. Thirty stage L3 nematodes were transferred from the maintenance petri dish with *Escherichia coli* OP50 to triplicate bacterial testing petri dishes with a titanium wire picker. The cocultures were incubated at room temperature for 5 days and observed for live/dead nematodes under a stereomicroscope (Zeiss).

Statistical analysis.

The results were expressed as means ± standard deviation. Data groups were compared using the one-way ANOVA and Student's t-test to evaluate associations between independent variables, and the *p* values were obtained. Three independent trials were conducted in triplicate for each experiment.

Results

Characteristics of the microbes found on dispensing nozzle of hand sanitizer dispensers

We sampled from the nozzle spout exits of fifty operational HSDs located in different places, ranging from clinical settings (hospitals and clinics) to commercial settings (restaurants and supermarkets) (Figure 1a). The HSDs originate from common brands used locally, where the hand sanitizers contain ethanol concentrations ranging from 60% to 75% (w/w). We found that nearly half of the HSDs contained microbial contaminants, where bacteria grew on LB agar, with a large range of bacterial numbers, ranging from 10^3 to 10^6 CFU/ml (Figure 1b). We picked and collected the unique colonies for initial evaluation using MALDI-TOF. Most bacterial isolates were identified as Gram-positive bacteria, where nearly 30% bacterial isolates were identified as *B. cereus*, while *Staphylococcus* species were next most frequently isolated (Figure 1c). Interestingly, the only Gram-negative bacterial species that we isolated was *E. cloacae* at 2%, which is an opportunistic pathogen associated with urinary tract infections and pneumonia in immunocompromised individuals [17]. Since *B. cereus* can form endospores which are resistant to alcohol, we evaluated if the HSD samples contain any endospores by using the endospore staining assay. We observed the presence of intact bacterial cells, but no endospores in the samples, indicating that the bacterial cells were vegetative (Supplementary Figure S1).

Microbes are tolerant to killing by low levels of alcohol

Based on their unique locations, such as hospital, clinic, school, supermarket and restaurant, we chose five *B. cereus* and two *E. cloacae* isolates for further microbiological evaluation. For *B. cereus*, we found that these isolates were tolerant to alcohol, where 3 isolates (BC1, BC2 and BC5) could survive 70% ethanol treatment (Figure 2). Since most commercial hand sanitizers contain at least 60% alcohol [18], this explains why *B. cereus* can colonize directly on the dispensing nozzle. Moreover, vegetative *B. cereus* could remain intact even with alcohol treatment (Supplementary Figure S2), indicating that the bacteria are tolerant to alcohol even in the absence of endospores. On the other hand, both *E. cloacae* isolates (EC1 and EC2) could not survive high concentrations of ethanol (Figure 2), where they were only unaffected by

17.5% alcohol. This could be attributed to prolonged ethanol evaporation from the nozzle that enables microbes to survive there [19].

Since bacteria spend most of their lives as biofilms on most biotic and abiotic surfaces with a high potential to contaminate environmental and food surfaces [20, 21], we also assessed if their biofilms could tolerate higher levels of alcohol. All bacterial isolates could grow biofilms on the peg lid of MBEC assay, where the *B. cereus* biofilms were in general tolerant to 70% alcohol (Figure 3). However, *E. cloacae* biofilms remain susceptible to high alcohol concentrations (Figure 3), which corroborate with our data of planktonic cells (Figure 2). This implied the possibility that *E. cloacae* were probably colonizing on HSDs with evaporated hand sanitizers.

Antibiotic resistance profiles of bacterial isolates

To evaluate if the HSD-associated bacteria are important in the context of public health and clinical settings, we first determined the antibiotic resistance profiles of the bacterial isolates, as determined according to the Clinical & Laboratory Standards Institute guidelines [11]. *B. cereus* were treated with the representative antibiotic of each class commonly used in clinical settings, where the isolates possessed resistance to beta-lactams and macrolides, but remain mostly sensitive to rifampicin, aminoglycosides and fluoroquinolones (Table 1). However, *E. cloacae* isolates were resistant to most common antibiotic classes, such as macrolides, beta-lactams, and Rifampicin (Table 1), indicating that HSDs could harbour multidrug resistant bacteria.

Bacterial isolates are virulent against Caenorhabditis elegans infection assay

The ability to cause disease is a major concern in public health. We evaluated the ability of the HSD-associated bacteria to infect and kill *C. elegans*, which is frequently used as an animal infection model to evaluate bacterial virulence [14, 15, 22]. For *B. cereus*, only BC1 and BC5 isolates were more virulent than the ATCC *B. cereus* strain, while the rest of the isolates were not virulent (Figure 4). Both EC1 and EC2 isolates were highly virulent against *C. elegans* (Figure 4), indicating that the HSD-associated bacteria could cause diseases in humans.

Discussion

HSDs are important for hygiene maintenance in clinical and public settings. Without proper hygiene and frequent maintenance of HSDs, HSDs might serve as potential breeding grounds for widespread dissemination of pathogens and antibiotic resistance, resulting in the spread of diseases across unknowing users. This has significant impact on human health as nearly half of all HSDs sampled from different locations, including hospitals and restaurants, possess bacteria in the dispensing nozzle. It is a surprising finding as the HSDs are automatic and contact-free with few opportunities for direct contact by users and hence microbial contamination. This is in contrast to manual HSDs that are highly susceptible to microbial contamination due to direct hand contact of the lever. Furthermore, contrary to assumptions that only spores could survive under harsh alcohol treatments, intact vegetative bacterial cells were present in the swabbed samples despite constant exposure to hand sanitizer in the nozzle spout. Hence, we suggest that there could be other factors that enable microbial colonization on automatic HSDs, such as prolonged ethanol evaporation from the nozzle [19], and misuse of HSDs, such as direct hand contact of nozzle spout.

Next, our work showed that HSDs-associated bacteria acquired some degree of alcohol tolerance, albeit restricted to a few bacterial isolates. While there were no signs of alcohol resistance in our study, some *B. cereus* isolates could survive the rapid killing of 70% alcohol at low viable numbers. Nonetheless, they were still a cause for concern, as *B. cereus* isolates were susceptible to low ethanol concentrations decades ago [23] and only their spores were resistant to ethanol [24]. This indicated that bacteria may evolve alcohol resistance in future with prolonged and overuse of alcohol disinfectants. Moreover, they were resistant to various antibiotic classes, with a heightened ability to cause disease. This showed that bacterial pathogens from HSDs possess alcohol tolerance, antibiotic resistance and virulence potential.

Our study has several limitations, where we employed culture-based techniques, instead of culture-independent methods, such as metagenomics, to identify HSD-associated bacteria. While there is a possibility of missing out on unculturable bacteria with fastidious nutrient requirements and anaerobic bacteria, many human pathogens can grow in microbiological agar and direct exposure to air enables the survival of aerobic bacteria. It is important to note that the HSD nozzles in direct contact with hand sanitizer were also exposed to the external environment with constant air circulation, indicating that anaerobic bacteria may not colonize well in such environments.

As we collected the samples over the course of one month, we also did not account for the changes in temperature and humidity of the surrounding environment, where such factors may alter the HSD-associated microbiome. Lastly, it is unclear how frequently the HSDs were utilized and maintained. A poorly maintained HSD which is rarely used, may encourage growth and colonization of microbes. Nonetheless, our work raises the need to consider how microbes can adapt to alcohol in infection prevention. From the manufacturers' point of view, the hand sanitizer formulations may require modifications to retain their effectiveness, such as using different alcohols such as propanol [25] or adding other antimicrobial compounds [26]. The HSD manufacturers may also consider using antimicrobial surfaces in the nozzle or incorporating UV light features to disinfect the nozzle after every use.

Conclusion:

HSDs are commonly assumed by the public to be sterile, but our work surprisingly showed that alcohol-tolerant microbes can exist on HSDs, even with direct contact with hand sanitizer. These microbes are pathogenic in nature, where they possess resistance to various antibiotic classes and virulence potential. This indicates that HSD-associated microbes may cause diseases in users, especially immunocompromised patients, the elderly and children. Hence, we propose frequent cleaning and replacing fresh hand sanitizers, if left unfinished over prolonged time. Public education is also key to proper use of such devices. These precautions will ensure protection of public health and sustainable use of sanitizing alcohols, thereby preventing the emergence of alcohol-resistant pathogens.

Acknowledgments:

YWSY, YM, SYL and WHP performed the experiments and analysed the data, while SLC planned the experiments and wrote the paper. All authors discussed the results and commented on the manuscript. All authors had access to all the data in the study and had final responsibility for the decision to submit to publication.

Conflict of interest statement

We declare no conflicting interests.

Funding statement

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Figures

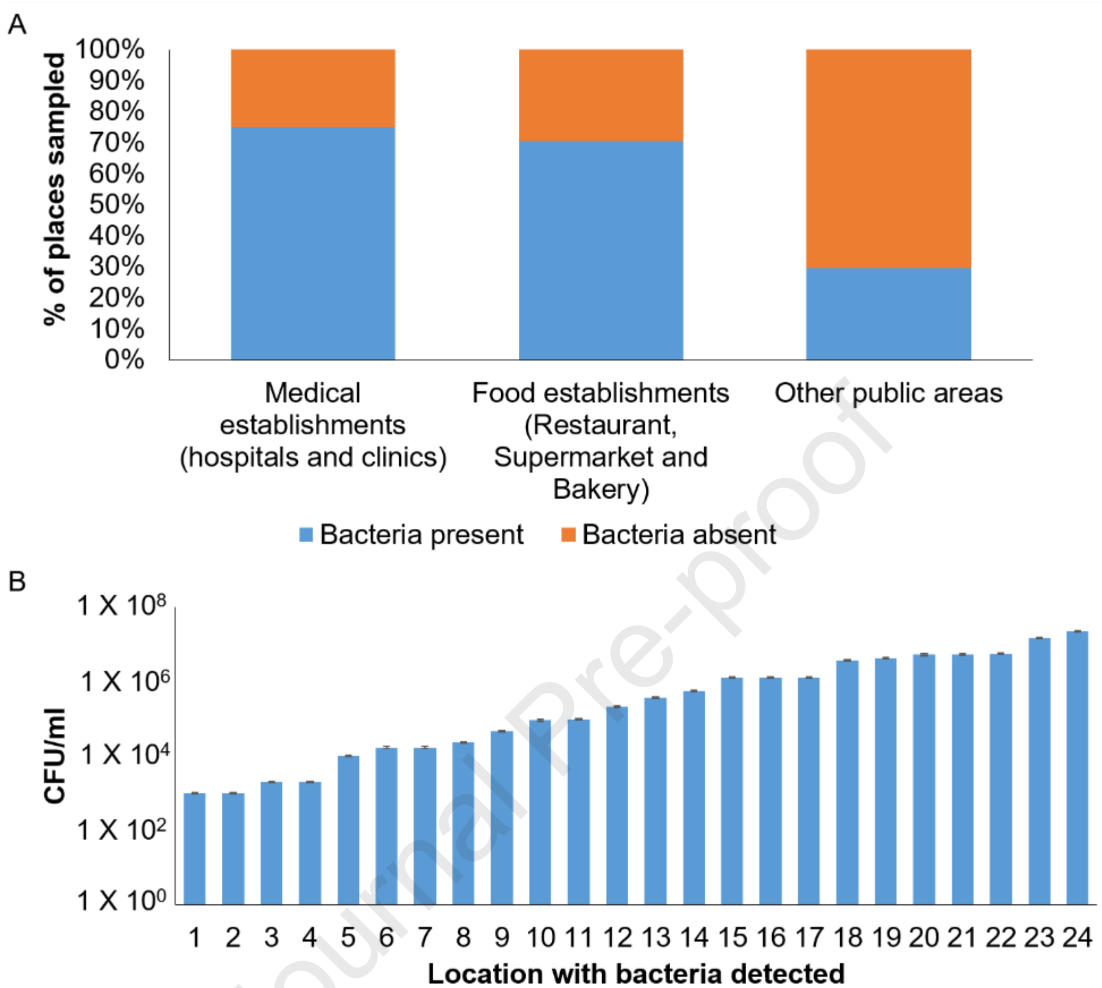

Figure 1. Characteristics of the microbes found on dispensing nozzle of hand sanitizer dispensers. (A) Study profile of samples collected from different locations for microbiological analysis. (B) Bacterial CFU from each dispenser contaminated with bacteria (presented in ascending order). Means and s.d. from triplicate experiments are shown.

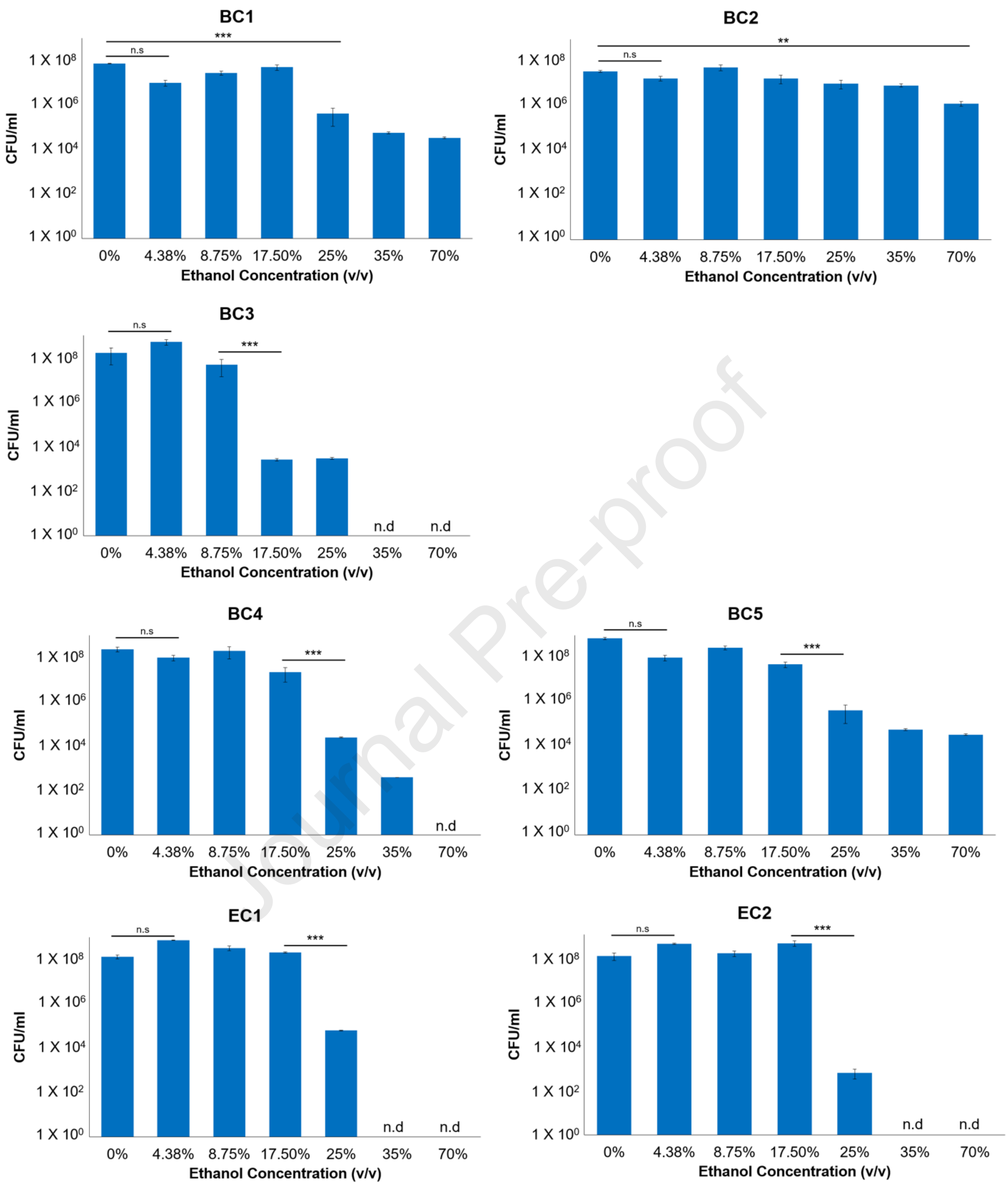

Figure 2. Microbes are tolerant to killing by low levels of alcohol after 10 mins of treatment. Means and s.d. from triplicate experiments are shown. BC: *Bacillus cereus*. EC: *Enterobacter cloacae*. *** states for p value < 0.001, n.s stands for not significant, n.d indicates not detectable.

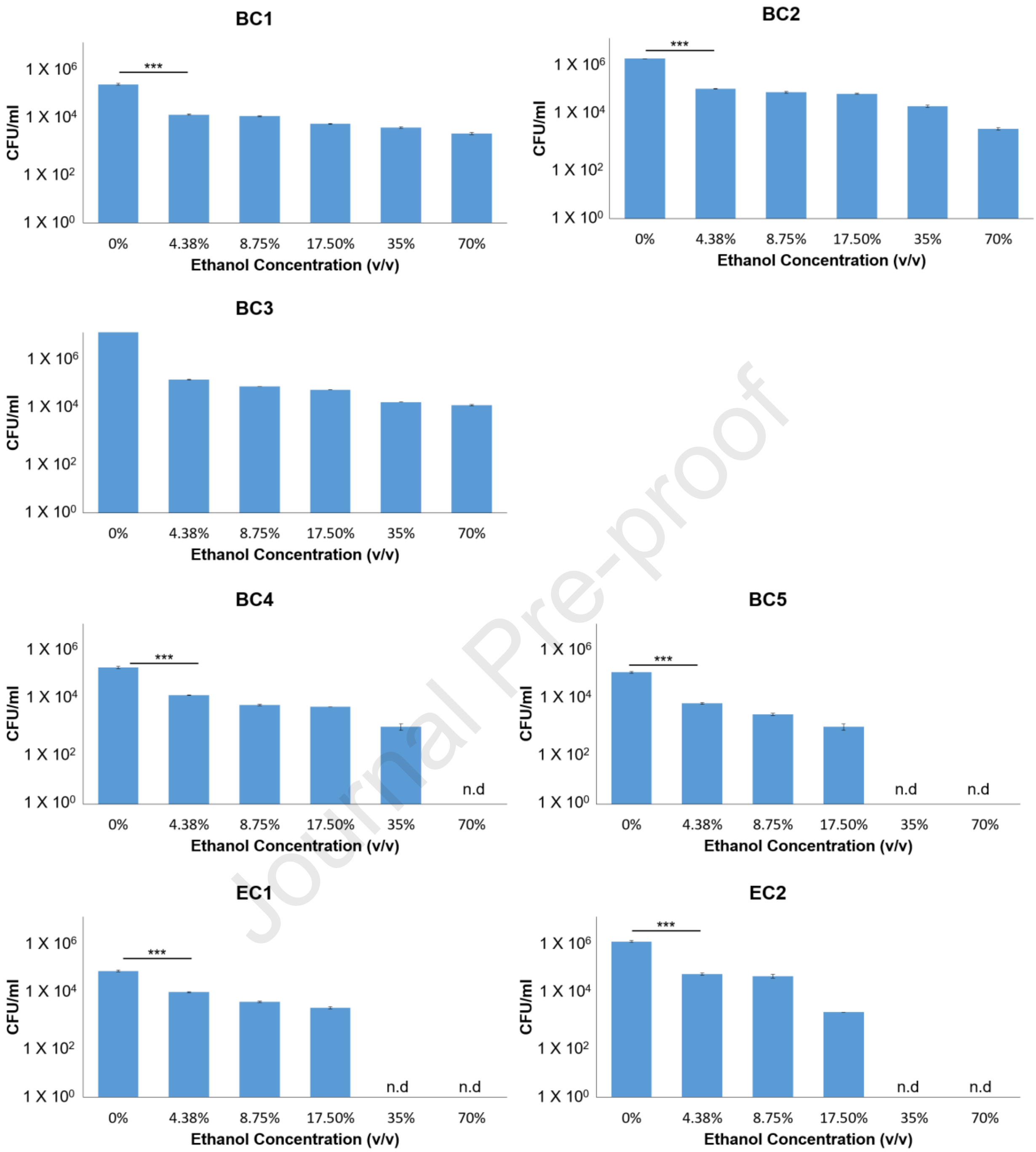

Figure 3. Microbial biofilms are tolerant to low levels of alcohol. Means and s.d. from triplicate experiments are shown. BC: *Bacillus cereus*. EC: *Enterobacter cloacae*. *** states for *p* value < 0.001, n.d indicates not detectable.

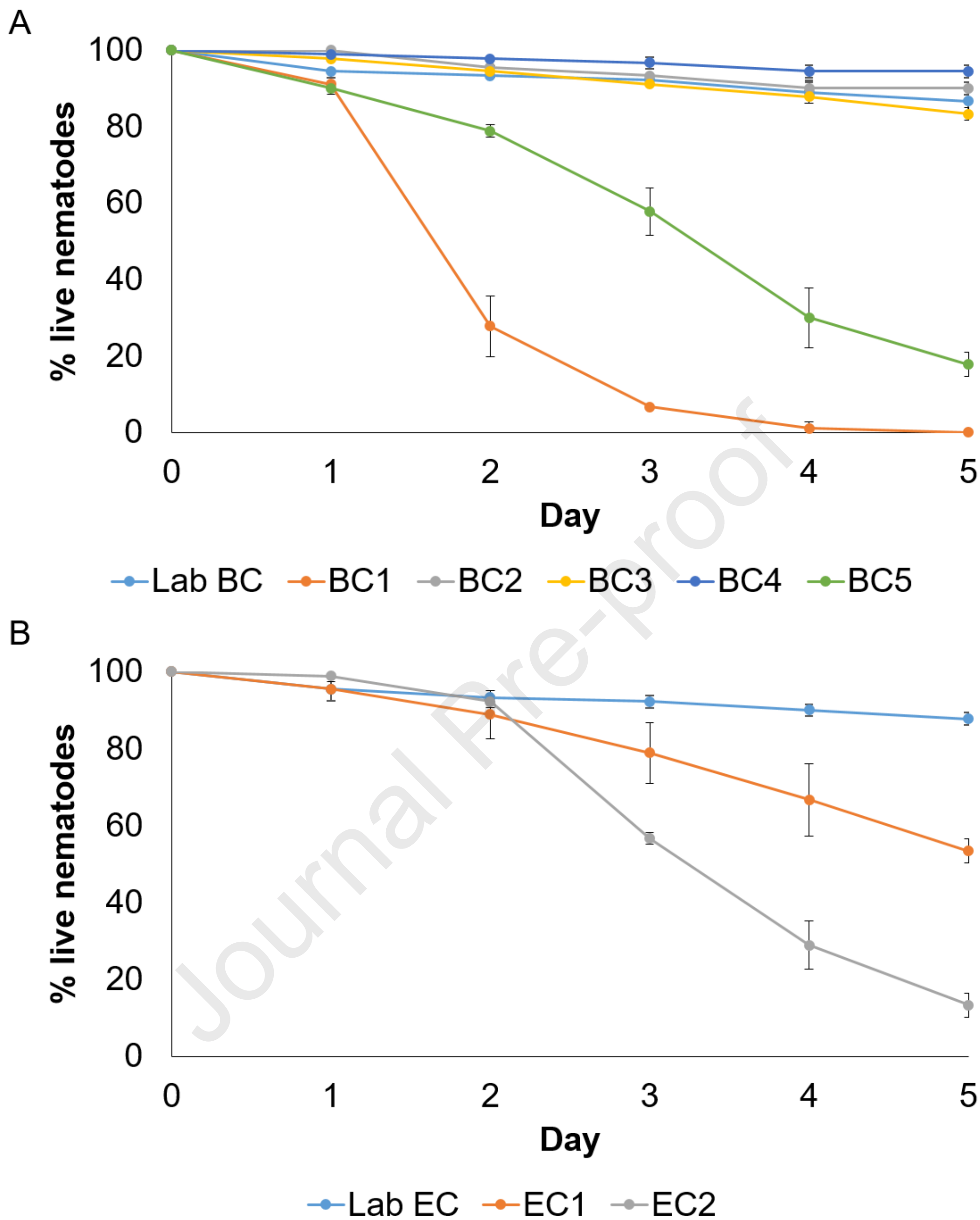

Figure 4. ***B. cereus*** **(BC) (A) and** ***E. cloacae*** **(EC) (B) are virulent against** ***Caenorhabditis elegans*** **infection assay**.

428 **Table 1.** Microbial species composition of isolates by MALDI-TOF.

Bacterial Species	% occurrence
Bacillus cereus	29
Staphylococcus warneri	9
Bacillus pumilus	6
Staphylococcus saprophyticus	5
Micrococcus luteus	3
Staphylococcus capitis	2
Enterobacter cloacae	2
Kocuria kristinae	2
Others	42

429

Table 2. Antibiotic resistance profiles of *B. cereus* (BC) and *E. cloacae* (EC) isolates. Minimal inhibitory concentrations (MICs) of Ampicillin (Amp), Gentamycin (Gm), Levofloxacin (Levo), Rifampicin (Rif), Erythromycin (Ery), and Amoxicillin (Amox) are listed, where their profiles are classified as S: sensitive; I: intermediate; R: resistant.

	Amp (ug/ml)	S/I/R	Gm (ug/ml)	S/I/R	Levo (ug/ml)	S/I/R	Rif (ug/ml)	S/I/R	Ery (ug/ml)	S/I/R
BC1	>1.00	R	2.00	S	0.31	S	0.60	S	5.00	I
BC2	>1.00	R	2.00	S	<0.15	S	0.60	S	5.00	I
BC3	>1.00	R	4.00	S	0.60	S	0.08	S	>10.00	R
BC4	>1.00	R	4.00	S	0.30	S	0.30	S	>10.00	R
BC5	>1.00	R	2.00	S	5.00	I	0.60	S	5.00	I

	Ery (ug/ml)	S/I/R	Levo (ug/ml)	S/I/R	Rif (ug/ml)	S/I/R	Amox (ug/ml)	S/I/R
EC1	>5.00	R	>0.08	S	>5.00	R	>10.00	R
EC2	>5.00	R	>0.08	S	>5.00	R	>10.00	R